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Hyoscyamus muticus L. subsp. *falezlez* methanolic extract: phytochemical composition and biological activities

Sofia Ayari-Guentri [1,2]*, Nadjette Djemouai [2,3,4], Somia Saad [2,5], Samira Karoune [5], Rabéa Gaceb-Terrak [2], Fatma Rahmania [2]

[1] Faculté des Sciences, Département des Sciences de la Nature et de la Vie, Université d'Alger 1, Benyoucef Benkhedda, 02. Didouche Mourad, Algiers, Algeria

[2] Laboratoire de Recherche sur les Zones Arides (LRZA), Faculté des Sciences Biologiques, Université des Sciences et de la Technologie Houari Boumediene (USTHB), BP32 El-Alia, 16111 Bab Ezzouar, Algiers, Algeria

[3] Laboratoire de Biologie des Systèmes Microbiens (LBSM), Ecole Normale Supérieure de Kouba, Algiers, Algeria

[4] Département de Biologie, Faculté des Sciences de la Nature et de la Vie et Sciences de la Terre, Université de Ghardaia, Ghardaïa, Algeria

[5] Centre de Recherche Scientifique et Technique sur les Régions Arides (CRSTRA), Biskra, Algeria

* Corresponding author e-mail: sofiaguentri.o@gmail.com

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ABSTRACT: This study aims to assess the phytochemical analysis and evaluate the antioxidant and antimicrobial activities of the methanolic extract obtained from the Algerian *Hyoscyamus muticus* L. subsp. *falezlez* leaves of Timimoun region. Methanolic extract of the plant contained the highest quantity of phenolics (148.00 ± 3.07 µg GAE/mg extract) and flavonoids (41.43 ± 0.90 µg QE/mg extract). The High-Performance Liquid Chromatography (HPLC) results showed dominance in the phenolic compounds: orientin, vitexin 2-O-rhamnoside and n-OH-cinnamic acid. Eight metabolites were identified and quantified by Gas Chromatography-Mass Spectrometry (GC-MS) which included five fatty acids, one dicarboxylic acid derivative, one bicyclic hydrocarbon and one fatty acid derivate. The GC-MS analysis revealed that palmitic acid (32.56%), linolenic acid (21.34%) and linoleic acid (11.24%) were the three major components. The methanolic extract showed an antioxidant activity for DPPH, ABTS, reducing power and phenanthroline assays. The strongest antioxidant activity was obtained with phenanthroline assay (value of $A_{0.5}$ <3.125 µg/mL). The antimicrobial investigation on thirteen microbial strains revealed that the methanolic extract showed low to moderate antibacterial activity against the Gram-positive and negative tested bacteria and no antifungal activity on all the tested fungi. This work suggests the use of leaves from *H. muticus* L. subsp. *falezlez* as a source of bioactive compounds with applications in the pharmaceutical, cosmetic and food industries.

Keywords: *Hyoscyamus muticus* L. subsp. *falezlez*; Phytoconstituents; Antioxidant potential; Antagonistic properties; HPLC; GC-MS.

1. INTRODUCTION

Medicinal plants synthesize various types of organic compounds that are categorized into primary and secondary metabolites. These secondary metabolites are synthesized from primary metabolites such as acetate, pyruvate, and amino acids [1]. They serve different purposes in plants, including growth regulation, allelopathy, an attractant for pollinator insects and defense against predators and infections [1, 2]. The secondary metabolites have exhibited interesting biological and pharmacological activities. In literature, more than 170.000 known secondary metabolites are reported and yet there's more to be discovered [2]. Primary (fatty acids) and secondary metabolites (phenolic compounds) are largely distributed in the plant kingdom. Furthermore, recent works suggest the potential health benefits of these compounds as antioxidants against oxidative stress diseases as well as many other disorders [3, 4].

The oxidative stress induced by the free oxygen radicals is one of the principal reasons for various chronic diseases such as cancer, gastric ulcers, diabetes, neurodegenerative and other disorders [5]. The human body possesses a protective system against the overproduction of these radicals however, when the protective system is insufficient, the antioxidant molecules are indispensable to counter-measure the excess free radicals [6]. Studies have shown that the strong activity of antioxidants is attributed to phytochemical compounds present in considerable amounts in plants [7].

In addition, several pathogenic species of bacteria and fungi, are causative agents of human and plant diseases. These diseases represent a critical problem to human health and the economy. The resistance developed by the pathogenic microorganisms against many antibiotics and antifungals restricts the choice of these molecules for therapy [8]. Therefore, the search for natural products from medicinal plants with antimicrobial activities is a field of scientific investigation [9].

Algeria is famous for its wealth of endemic medicinal plants belonging to various families [10]. *Hyoscyamus* is one of the most important genera of the Solanaceae family comprising about 85 genera and more than 2800 species worldwide [11]. The phytochemical analysis showed that *Hyoscyamus* species contained alkaloids, terpenes, flavonoids, tannins, saponins, carbohydrates and anthraquinones. They exerted many biological effects including antioxidant, antibacterial, antispasmodic, anesthetic, sedative, anticholinergic and analgesic properties [4, 12-17].

In Algeria, one of the plant species growing in sandy areas is *Hyoscyamus muticus* L. commonly known in English as Egyptian Henbane and its vernacular name is Habala in the Timimoun region [18,19]. In Algerian traditional medicine, the aerial parts of this plant prepared as decoction are used to treat articular pains and kidney diseases [20]. This species is of high economic importance for its tropane alkaloids (hyoscyamine), which are used in medicine because of their analgesic, anticholinergic, antispasmodic, mydriatic, and sedative activities [13]. As a cultural resource, apart from its medicinal value, *Hyoscyamus* is and has been used deliberately in ancient and traditional societies as a poison and as a hallucinogen in rituals [21, 22]. Although the studied plant has economic and therapeutic values, only two studies were published on *H. muticus* L. and the subspecies *H. muticus* L. subsp. *falezlez* [19, 23]. *H. muticus* L. is a wild or spontaneous species and is cultivated in Egypt, India, Pakistan and USA for the production of the medicinally important tropane alkaloids hyoscyamine and scopolamine. [24]. Furthermore, the Algerian authorities provide great importance to the protection of this valuable species [25]. Within the frame of investigating medicinal plants from arid regions of Algeria, this study aimed to accomplish a phytochemical analysis of *H. muticus* L. subsp. *falezlez* leaves extract and to investigate its antioxidant and antimicrobial activities.

2. MATERIALS AND METHODS

2.1. Collection, identification and preparation of extracts

H. muticus L. subsp. *falezlez* leaves were collected in June of 2021 from Institut National de la Recherche Agronomique (INRA station) at Timimoun Province (South West of Algeria). The identification and authentication of the plant were determined using the description established by Quezel and Santa (1963) [10]. The voucher specimen (MP.19.8.13.6/2021) was deposited in the Herbarium of Laboratoire de Recherche sur les Zones Arides (USTHB, Algeria).

The collected leave samples (3 kg) were dried at room temperature in the shade. Then, the plant materials were grounded into a fine powder using an electrical grinder. Extraction from the leaves was performed using a Soxhlet extractor. Two grams of powdered leaves were placed into a Whatman paper cartridge and extracted with 200 mL of methanol. The process of extraction continued for 4 h at the boiling point of the methanol (65 °C). Finally, the solvent was evaporated at 40 °C in a rotary evaporator (BUCHI) to obtain the crude extract. The yield of extraction was calculated using the following formula:

Extraction yield (%) = [Weight of dry extract (g)/Weight of the sample used for the extraction (g)]×100

The extract was stored at -16 °C pending further experiments.

2.2. Quantitative phytochemical analysis

2.2.1. Total phenolic content

The total phenolic content (TPC) of the crude extract was determined according to the Folin-Ciocalteu method, as described by Singleton et al. [26]. A volume of 20 µL of the methanolic extract was added to 100 µL of Folin-Ciocalteu reagent (10-fold dilutions) and 75 µL of sodium carbonate solution (7.5%). After 2 h of incubation at room temperature, the absorbance was measured at 765 nm using a 96-well microplate reader (Perkin Elmer, EnSpire, Singapore). The TPC was expressed as Gallic Acid Equivalent per gram (GAE/g) of the plant extract.

2.2.2. Total flavonoid content

The total flavonoid content (TFC) was quantified using the aluminum trichloride ($AlCl_3$) assay [27]. Briefly, 50 µL of the methanolic extract was mixed with 50 µL of $AlCl_3$ solution (10%) followed by the addition of 150 µL of sodium acetate solution (10%). The mixture was allowed to stand for 2.30 h at room temperature. The absorbance was measured using the 96-well microplate reader at 440 nm. The TFC was expressed as Quercetin Equivalent per gram (QE/g) of the plant extract.

2.3. Determination of phenolic compounds by HPLC

The separation by High-Performance Liquid Chromatography (HPLC) was achieved using an Agilent model 1100 instrument equipped with a quaternary gradient pump, a thermostated column compartment, a manual injector and a Diode Array Detection system (DAD). The column that was used is a C18 with dimensions of 250 x 4.6 mm; 5 µm (Hypersil BDS) and thermostated at 30 °C. The solvents were (A) ultrapurified water/acetic acid (0.2%) (v/v) and (B) acetonitrile. The gradient was linear at a flow rate of 1 mL/min, from 95% of solvent A to 100% of solvent B for 30 min. DAD was performed from 200 nm to 400 nm. The injection volume was 5 µL for the methanolic extract and standards.

2.4. Determination of compounds by GC-MS

The chemical composition of the methanolic extract was investigated by Gas Chromatography-Mass Spectrometry (GC-MS) analysis. The sample was methylated according to the method reported by Goren et al. [28]. The methyl ester components were analyzed using a Perkin Elmer Clarus 500 apparatus. The column was 5-MS (30 m x 0.25 mm, 0.25 µm film thickness). The oven temperature was programmed as the initial temperature was 90 °C (during 1 min), then increased at the rate of 6 °C/min to 220 °C (during 0 min), 10 °C/min to 290 °C (during 1.23 min) and finally increased further at the rate of 40 °C/min to 310 °C (during 7.5 min). Carrier gas was helium (1 mL/min). One microliter (1 µL) of the sample was injected into the system in split mode. The injector temperature was 250 °C. Mass spectra were recorded with Electron Ionization (EI) mode at 70 eV and the spectral range was 20-550 m/z. The transfer line temperature and source temperature were 250 °C.

The compounds were identified by comparing their retention times and their mass spectral data with two available databases of the National Institute of Standards and Technology (NIST 09) and WILEY mass spectral library data provided by the software of the GC-MS system [29].

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical scavenging activity of the methanolic extract was measured according to the method described by Blois (1958) [30] with some minor modifications. Forty microliters (40 µL) of the sample were prepared in methanol at different concentrations (12.5 - 800 µg/mL) and then, were mixed with 160 µL of a methanol solution of DPPH. The reaction mixture was incubated in the dark at room temperature for 30 min. The absorbance of the resulting solutions was measured at 517 nm using the 96-well microplate reader. The inhibition activity was calculated using the formula:

$\%\ \text{Inhibition} = [(A_0 - A_1/A_0)] \times 100$

Where A_0 is the absorbance value of the control and A_1 is the absorbance of the sample. BHT was used as a positive control. The results were also expressed as the IC_{50} (µg/mL) that was determined graphically by linear regression.

2.5.2. ABTS radical scavenging activity

The ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging activity of the methanolic extract was assessed as described by Re et al. (1999) [31]. The solutions of 7 mM ABTS and 2.45 mM potassium persulphate were prepared using distilled water. Afterward, the two solutions were mixed and incubated at room temperature for 16 h in the dark. The subsequent solution was diluted in ethanol to give an absorbance of 0.70 ± 0.02 at 734 nm. Forty microliters (40 µL) of the sample were prepared in methanol at different concentrations (12.5 - 800 µg/mL) and then, were added to 160 µL of ABTS diluted solution and incubated for 10 min. BHT was used as a positive control and the absorbance was measured in the 96-well microplate reader at 734 nm. The results were expressed as inhibition percentage (%) and the IC_{50} (µg/mL) value was calculated by linear regression analysis.

2.5.3. Reducing power

The reducing power method was applied with some modifications based on the work of Oyaizu (1986) [32]. A volume of 10 µL of the sample was prepared in methanol at different concentrations (3.125-200

µg/mL), then, was mixed with 40 µL of 0.2 M phosphate buffer (pH 6.6) and 50 µL of potassium ferricyanide solution (1%). The mixture was incubated at 50 °C for 20 min. Afterward, 50 µL of (10%) trichloroacetic acid solution, 40 µL of distilled water and 10 µL of ferric chloride (0.1%) were added to the mixture. The absorbance was measured in the 96-well microplate reader at 700 nm. Ascorbic acid standard solutions were used to construct analytical curves and the $A_{0.5}$ values were calculated.

2.5.4. Phenanthroline assay

The method reported by Szydlowska-Czerniaka et al. [33] was used to determine the antioxidant capacity test of phenanthroline. Ten microliters (10 µL) of the sample were prepared in methanol at different concentrations (3.125-200 µg/mL) and were added to 50 µL of ferric chloride (0.2%), 30 µL of phenanthroline solution (0.5%) and 110 µL of methanol. The obtained solutions were incubated in the dark at 30 °C for 20 min. The absorbance was determined at 510 nm using the 96-well microplate reader. The BHT was used as the antioxidant standard for the comparison of activities.

2.6. Antimicrobial activity

The antimicrobial activity of the methanolic extract of *H. muticus* L. subsp. *falezlez* was assessed against four clinical bacterial strains: *Staphylococcus aureus* (ATCC 43300), *Listeria monocytogenes* (ATCC 13932), *Pseudomonas aeruginosa* (ATCC 7029) and *Escherichia coli* (ATCC 8739), one yeast *Candida albicans* (M3) and eight fungal strains: *Aspergillus carbonarius* (M333), *Aspergillus westerdijkiae* (ATCC 3174), *Aspergillus brasiliensis* (ATCC 16404), *Penicillium expansum* (Pe), *Umbelopsis ramanniana* (NRRL 1829), *Fusarium graminearum* (Fg), *Fusarium oxysporum* f.sp. *albedinis* (Foa) and *Fusarium culmorum* (Fc). All these strains were obtained from the microbial collection of LBSM Laboratory. The antimicrobial activities of the obtained extract were assessed by the disk diffusion method [34]. The methanolic extract was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 200 µg/mL. Twenty microliters (20 µL) of the extract were loaded into sterile filter paper disks. These disks were placed on Muller-Hinton agar and Potato-Dextrose Agar plates previously seeded with 100 µL of bacterial and fungal inocula (0.5 Mac Farland), respectively [35]. Then, all Petri dishes were incubated at 37 °C (24 h) for bacteria and 25 °C (48 h) for fungi. DMSO added discs were tested as a negative control. The antimicrobial activity was estimated after measurement of diameters of zones of inhibition in mm.

2.7. Statistical analysis

The parameters taken into consideration in our study were carried out in triplicates from which the mean values and their respective Standard Deviations (SD) were calculated. Significant differences were calculated after the performance of one-way analysis of variance (ANOVA) test. Means were compared by Least Significant Difference (LSD) multiple Duncan's range test with differences considered to be significant at ($p < 0.05$). Correlation between the results of yield, TPC, TFC, DPPH, ABTS, reducing power and phenanthroline were determined. Results were considered statistically significant when *p*-values were below 0.05. Statistical tests were performed using STATISTICA software (version 6.0, 2001).

3. RESULTS

3.1. Yield and quantitative phytochemical analysis

In the present study, the yield of the leaves methanolic extract of *H. muticus* L. subsp. *falezlez* was 75.04% ± 0.10 (w/w). Total phenolic and flavonoid contents in the methanolic extract of *H. muticus* L. subsp.

falezlez were determined and the obtained results are shown in Figure 1. The content of total phenolic was high with a value of 148.00 ± 3.07 µg GAE/mg extract followed by the total flavonoids with a content of 41.43 ± 0.90 µg QE/mg extract.

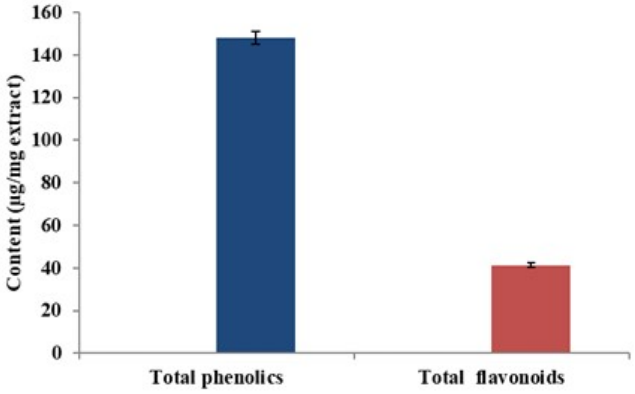

Figure 1. Quantitative analyses of total phenolic (µg GAE/mg extract) and total flavonoid (µg QE/mg extract) contents of the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez.* data are expressed as mean (*n= 3*) ± SD.

3.2. Identification of phytoconstituents by HPLC

The composition of the methanolic extract was summarized in Table 1 and the chromatograms that were recorded at 280 nm and 300 nm are shown in Figure 2.

Table 1. Phenolic compounds identified by High-Performance Liquid Chromatography of the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez*.

Compound number	Retention time (min)	Identified compound	Compound class
1	3.286	Gallic acid	Phenolic acid
2	3.681	Hydroxy-quinone	Quinone
3	5.334	Resorcinol	Diphenol
4	6.524	Resorcylic acid	Phenolic acid
5	6.994	Vanillic acid	Phenolic acid
6	7.239	Syringic acid	Phenolic acid
7	7.941	Orientin	Flavonoid
8	8.623	Vitexin 2-O-rhamnoside	Flavonoid
9	8.750	n-OH-cinnamic acid	Phenolic acid
10	9.020	Rutin	Flavonoid
11	9.347	Ferulic acid	Phenolic acid
12	9.587	Luteolin-7-glycoside	Flavonoid
13	9.844	Salicylic acid	Phenolic acid
14	10.504	Riboflavin	Vitamin
15	10.647	3,4,5-trimethoxybenzoic acid	Phenolic acid
16	11.777	m-anisic acid	Phenolic acid
17	12.892	Luteolin	Flavonoid
18	13.638	Cinnamic acid	Phenolic acid
19	14.376	Apigenin	Flavonoid

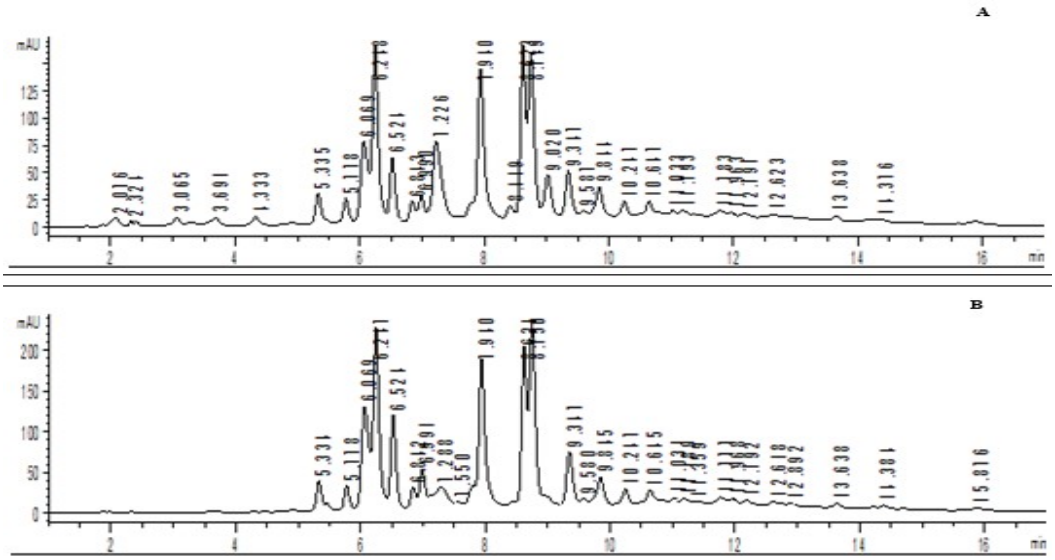

Figure 2. HPLC chromatograms of the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez* at A: 280 nm and B: 300 nm.

The peaks were identified by their retention times and UV-Vis spectra in the sample and standards. The HPLC profiles of the methanolic extract showed several peaks corresponding to different phenolic compounds with quantitative and qualitative differences. We noticed that the peaks of major phenolic compounds were observed at 5 to 10 min (Figure 2 A and B). A total of 19 compounds were identified that belonged to phenolic acids (10), flavonoids (6) and other phenolic compounds (3). The identified phenolic acids were n-OH-cinnamic, syringic, ferulic, salicylic, gallic, resorcylic, vanillic, 3,4,5-trimethoxybenzoic, m-anisic and cinnamic acids. For flavonoids, the six compounds that were identified are C- or O-glycosyl-flavonss (orientin, vitexin 2-O-rhamnoside and luteolin-7-glycoside), flavone (luteolin and apigenin) and flavonol (rutin). The other identified phenolic compounds were resorcinol, hydroxy-quinone and riboflavin.

Based on the abundance, the major compounds and their retention times that were identified in the methanolic extract were orientin (7.941 min), vitexin 2-O-rhamnoside (8.623 min) and n-OH-cinnamic acid (8.750 min) followed by average quantities of syringic acid (7.239 min), rutin (9.020 min), ferulic acid (9.347 min) and salicylic acid (9.844 min). At last, the remaining compounds were identified as minor compounds.

3.3. Identification of phytoconstituents by GC-MS

The phytoconstituents contained in the methanolic extract of *H. muticus* L. subsp. *falezlez* leaves were detected by GC-MS. The GC-MS spectrum showed the presence of several peaks with different retention times (Figure 3).

Based on the results mentioned in Table 2 and Figure 3, eight compounds were identified in the methanolic extract of *H. muticus* L. subsp. *falezlez* leaves with the following proportions: palmitic acid (32.56%), linolenic acid (21.34%), linoleic acid (11.24%), stearic acid (8.74%), pentane dioic acid (2,4-di-t-butylphenyl) ester (6.54%), 3-(2,3-dihydro-1H-inden-1-yl)-2-methyl-2-cyclopenten-1-one (2.40%), myristic acid (0.84%), octacosa-12,14,16-triene-10,18-diynedioic acid, (0.52%). The total of saturated methyl esters fatty acids was 42.14% while the total of polyunsaturated methyl esters fatty acids was 32.58%.

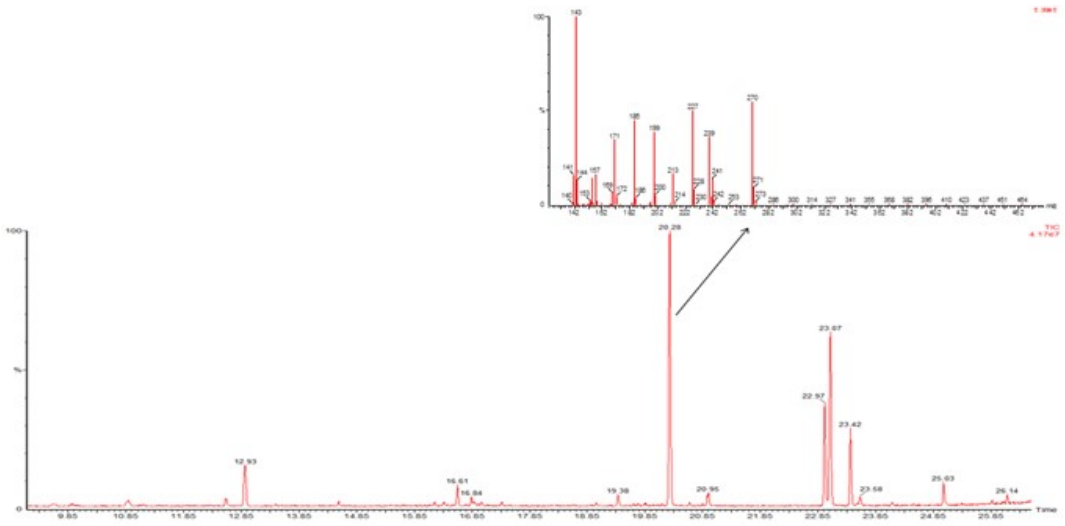

Figure 3. A typical GC-MS chromatogram of compounds present in the methanolic extract and the mass spectrum of the main pick from leaves of *Hyoscyamus muticus* L. subsp. *falezlez*.

3.4. Antioxidant activity

The antioxidant activity of leaves methanolic extract of *H. muticus* L. subsp. *falezlez* was tested by DPPH, ABTS, reducing power and phenanthroline assays. The obtained results from the different assays were depicted in Table 3.

DPPH and ABTS assays are two of the used tests for the evaluation of the free-radical scavenging effects. In these assays, free radicals will be scavenged by antioxidant molecules resulting in a decrease in absorbance. The IC_{50} value of the DPPH test was found to be 135.54 ± 2.00 µg/mL for our analyzed extract which was higher than BHT (IC_{50} = 34.54 ± 1.60 µg/mL). Thus, we conclude that BHT had an important antioxidant activity than our methanolic extract. In ABTS radical scavenging assay, the IC_{50} of 36.54 ± 0.20 µg/mL was determined for the methanolic extract and it was compared with the standard BHT (IC_{50} <12.5 µg/mL). So, BHT had also an important antioxidant activity than our analyzed methanolic extract.

The reducing power and phenanthroline antioxidant capacities were used to investigate the ability of *H. muticus* L. subsp. *falezlez* extract for reduction of metallic ions. The methanolic extract possessed a value of $A_{0.5}$ (59.19 ± 2.70 µg/mL) in the reducing power which was less efficient than the ascorbic acid ($A_{0.5}$ = 6.52 ± 0.07 µg/mL). Analysis of metal iron-reduction assessed by phenanthroline test showed that the methanolic extract of leaves (<3.125 µg/mL) was more efficient than the BHT (9.71 ± 0.90 µg/mL) (Table 3).

The results showed that the methanolic extract from leaves of *H. muticus* L. subsp. *falezlez* exhibited interesting antioxidant effects. *H. muticus* L. subsp. *falezlez* extract was found to be a high scavenging agent against DPPH and ABTS free radicals. Moreover, the methanolic extract showed notable antioxidant properties on ions reduction.

Table 2. Compounds identified by Gas Chromatography-Mass Spectrometry in the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez*.

Compound number	Compound name	Compound class	Retention time (min)	Molecular formula	Molecular weight	Area (%)	Biological activity	References
1	Pentane dioic acid (2,4-di-t-butylphenyl) ester	Dicarboxylic acid derivative	12.93	$C_{19}H_{28}O_4$	320	6.54	/	/
2	3-(2,3-dihydro-1h-inden-1-yl)-2-methyl-2-cyclopenten-1-one	Bicyclic hydrocarbon	16.61	$C_{15}H_{16}O$	212	2.40	/	/
3	Myristic acid	Fatty acid	16.85	$C_{14}H_{28}O_2$	242	0.84	Antioxidant and antidiabetic activities	[54]
4	Octacosa-12,14,16-triene-10,18-diynedioic acid	Fatty acid derivate	17.38	$C_{28}H_{40}O_4$	440	0.52	/	/
5	Palmitic acid	Fatty acid	20.28	$C_{16}H_{32}O_2$	270	32.56	Antimicrobial, antidiabetic and cytotoxic activities	[9]
							Anti-inflammatory and antifibrotic activities	[51]
6	Linolenic acid	Fatty acid	22.97	$C_{18}H_{30}O_2$	294	11.24	Antifungal and antioxidant activities	[52]
7	Linoleic acid	Fatty acid	23.07	$C_{18}H_{32}O_2$	292	21.34	Antifungal and antioxidant activities	[52]
8	Stearic acid	Fatty acid	23.41	$C_{18}H_{36}O_2$	298	8.74	Antibacterial activity	[9]
							Antifungal activity	[52]
							Antidiarrheal and antiproliferative activities	[53]

Table 3. Antioxidant activity of the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez*.

Extract/standard	IC_{50} (µg/mL)		$A_{0.5}$ (µg/mL)	
	DPPH	ABTS	Reducing power	Phenanthroline
Methanolic extract	135.54 ± 2.00[b]	36.54 ± 0.20[b]	59.19 ± 2.70[b]	<3.12
BHT	34.54 ± 1.60 [a]	<12.50[a]	ND	9.71 ± 0.90[a]
Ascorbic acid	ND	ND	6.52 ± 0.07[a]	ND

Values are mean of three replicates ($n = 3$) ± SD. Results with different superscript letters are significantly different ($p \leq 0.05$). IC_{50} (mg/mL): Concentration at which 50% is inhibited. $A_{0.5}$ (mg/mL): Concentration indicating 0.50 absorbance intensity. BHT: Butylated hydroxytoluene, ND: Not determined.

3.5. Antimicrobial activity

In vitro antimicrobial activities of the methanolic extract of *H. muticus* L. subsp. *falezlez* were assessed by evaluating the inhibition zones. The results are summarized in Table 4. The methanolic extract was found to be active against all the tested bacteria. The inhibition diameter values varied depending on the species of bacteria and ranged from 7.83 ± 0.29 mm to 15.17 ± 0.29 mm. The results of the antibacterial effect revealed that the methanolic extract showed moderate activity against *E. coli* followed by *P. aeruginosa* and *L. monocytogenes* while a week activity was recorded for *S. aureus*. Furthermore, no antifungal activity was observed for the methanolic extract.

Table 4. Antimicrobial activity of the methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez* based on the disc diffusion assay.

Microorganism	Diameter inhibition in 200 µg in mm for the methanolic extract
Staphylococcus aureus (ATCC 43300)	07.83 ± 0.29
Listeria monocytogenes (ATCC 13932)	12.33 ± 0.58
Pseudomonas aeruginosa (ATCC 7029)	14.00 ± 0.00
Escherichia coli (ATCC 8739)	15.17 ± 0.29
Candida albicans (M3)	-
Aspergillus carbonarius (M333)	-
Aspergillus westerdijkiae (ATCC 3174)	-
Aspergillus brasiliensis (ATCC 16404)	-
Penicillium expansum (Pe)	-
Umbelopsis ramanniana (NRRL 1829)	-
Fusarium graminearum (Fg)	-
Fusarium oxysporum f.sp. *albedinis* (Foa)	-
Fusarium culmorum (Fc)	-

Values are the mean of three replicates ($n = 3$) ± SD; (-) no inhibition zone.

3.6. Correlation between total phenolic and flavonoid contents, antioxidant and antimicrobial activities

In order to analyze the relationship correlation between the total phenolic and flavonoid contents, antioxidant and antimicrobial activities of methanolic extract from leaves of *Hyoscyamus muticus* L. subsp. *falezlez*, Pearson's correlations were applied (Table 5).

Table 5. Matrix of correlation between yield, TPC, TFC, antioxidant and antimicrobial assays.

	TPC	TFC	DPPH	ABTS	RP	Phen	SA	LM	PA	EC
Yield	-1.00*	-0.95	0.87	-0.55	-0.30	-1.0*	-0.94	-0.48	1.0*	-1.0*
TPC		0.97	-0.84	0.61	0.23	1.0	0.91	0.42	-1.0	1.0
TFC			-0.68	0.78	-0.02	0.9	0.78	0.18	-0.9	0.9
DPPH				-0.07	-0.72	-0.9	-0.99	-0.85	0.9	-0.9
ABTS					-0.63	0.5	0.22	-0.47	-0.5	0.5
RP						0.3	0.61	0.98	-0.3	0.3
Phen							0.94	0.50	-1.0*	1.0*
SA								0.76	-0.9	0.9
LM									-0.5	0.5
PA										-1.0*

*Significant correlation with p <0.05. TPC: total phenolic content, TFC: total flavonoid content, RP: reducing power, Phen: phenanthroline, antimicrobial effect against SA: *Staphylococcus aureus*, LM: *Listeria monocytogenes*, PA: *Pseudomonas aeruginosa*, EC*: Escherichia coli*.

Results showed a perfect positive relationship between the extraction yield and the antibacterial activity evaluated against *Pseudomonas aeruginosa* (r = 1). On the other hand, a perfect negative relationship was found between the antioxidant activity assayed by phenanthroline test, the antibacterial activity evaluated against *Escherichia coli* and yield (r = -1). The total phenolic and flavonoid contents were positively and strongly correlated with the antibacterial activity, ABTS and phenanthroline assays.

4. DISCUSSION

The results show that the extraction yield as well as the phenolic compounds content are high. The high value in terms of yield can be explained by the polar nature of the used methanol extraction solvent which facilitates the solubilization of different metabolites [36]. So, total flavonoids and total phenolics were produced in important quantities in this plant extract. These compounds have a principal role in the filtration of the UV rays thus, representing a photoprotective mechanism for the plant and a role in protection against the phytopathogenic agents.

In comparison with other previous studies, the phytochemical screening of the aerial parts of *H. muticus* from Saudi Arabia showed the richness of the plant in total phenolics and flavonoids and the absence of tannins when using ethanol extraction as was published by Mohd and Nudrat [37]. Furthermore, Elsharkawy et al. [15] reported the richness of the aerial parts of *H. muticus* in phenolics, flavonoids and tannins after methanol extraction. However, Kebaili et al. [23] reported that the phytochemical screening of the hydroalcoholic extract of the same plant from Djanet (Algeria) showed the presence of flavonoids and tannins.

Our results were different from those obtained by Al-Tohamy et al. [14] where they reported total phenolic and flavonoid contents of 20 mg GAE/g extract and 8 mg QE/g extract, respectively for the methanolic extract of *H. muticus* collected from Egypt.

Other studies that were carried out on different species of the genus *Hyoscyamus* revealed the presence of phenolic compounds in significant amounts for their different studied parts [16, 17, 38-40]. Phytochemicals are important because they have many biological activities including antioxidant properties [41].

Phenolic compounds are the most abundant family of phytoconstituents. These compounds are known for their several aromatic rings and hydroxyl groups and their roles in defense mechanisms against pathogenic microorganisms as well as plant growth and reproduction [42]. On the other hand, flavonoids are a group of phytochemicals that are diverse and generally associated with the reduction of major chronic disease risks in humans [43].

Several biological and pharmacological activities have been attributed to different flavonoids. The major compound found in our methanolic extract after analysis by HPLC was orientin. This last was reported to possess many activities such as antioxidant, antiviral, antibacterial, radiation protective, neuroprotective, antidepressant-like, antiadipogenesis, antithrombotic, antiplatelet activities and others [44-46]. For the second major compound, vitexin showed antioxidant activity, anti-inflammatory, antihyperalgesic, antinociceptive, antiepileptic, anticonvulsant, antidepressant, neuroprotective effects and anti-Alzheimer's disease [47]. Also, hydroxycinnamic acid was found to have antioxidant, antimicrobial, antiviral and anticancer properties [48-49].

This phytochemical composition is described for the first time for the Soxhlet methanolic extract of *H. muticus* L. subsp. *falezlez*. Nevertheless, extraction by the method of Lebreton revealed the presence of some phenolic acids and flavonoids like caffeic, ferulic, trans-cinnamic acids and quercetin for *H. muticus* L. subsp. *falezlez* [19]. In addition, phenolic compounds were found in *H. muticus* methanolic extract such as ferulic acid, methyl salicylate and methyl ferulate [15]. On the other hand, *H. niger* and *H. reticulatus* in the study of Jassbi et al. [40] showed the presence of quercetin-3O-glucoside-rhamnoside-rhamnoside, rutin and chlorogenic acid.

GC-MS analysis is a tool used for the separation and identification of phytocompounds [50]. The methanolic extract of *H. muticus* L. subsp. *falezlez* was subjected to GC-MS analysis with the spectrum confirming the presence of many bioactive compounds in leaves extract of *H. muticus* L. subsp. *falezlez* with different retention times. The gas chromatogram shows the presence of relative concentrations of numerous compounds present in *H. muticus* L. subsp. *falezlez* getting eluted at different retention times.

In terms of abundance, palmitic acid was the major compound in the analyzed methanolic extract. This compound is known for its biological activities including antimicrobial, antidiabetic, cytotoxic, anti-inflammatory and antifibrotic [9, 51]. The linoleic and linolenic acids have been reported to have antifungal and antioxidant activities [52]. Stearic acid was found to have antifungal and antibacterial activities [9, 52], antidiarrheal and antiproliferative properties [53]. According to Alonso-Castro et al. [54], myristic acid has antioxidant and antidiabetic effects. Our results corroborate those obtained by Ramadan et al. [4], in their study where they showed that *H. muticus* and *H. niger* seed oils were rich in linoleic, oleic and palmitic acids. The obtained results are in line with Keskin et al. results [55] where they showed that the main fatty acids identified in the vegetable oil of the aerial parts of *H. albus*, *H. aureus*, *H. reticulatus* and *H. leptocalyx* were palmitic, linoleic, linolenic and oleic acids. However, our results are different from those obtained by Guler [16], who revealed that the obtained oil from *H. reticulatus* aerial parts was rich in lauric, capric and undecanoic acids and the percentages of oleic, linoleic and palmitic acids were low with 1.81%, 4.34% and 7.70%, respectively. When comparing our results with those of literature, we noted that previous studies showed that species of the genus *Hyoscyamus* had high amounts of polyunsaturated fatty acids [4, 55].

Antioxidants were reported from medicinal plants and thus can be valuable for human disease treatments [56]. In this study, the antioxidant properties of methanolic extract of leaves of *H. muticus* L. subsp. *falezlez* were ascertained with the help of DPPH, ABTS, reducing power and phenanthroline assays.

The obtained results show that the methanolic extract presented a good antioxidant activity with all the used tests. We also noted that the best antioxidant activity was obtained with the phenanthroline test. Our results corroborate the previous reports on several other plant species of the same genus [4, 15, 16, 19].

The secondary metabolites like phenolics and flavonoids from plants have been stated to be potent free-radical scavengers; they are found in all parts of the plant such as leaves, fruits, seeds and roots [57-59]. These results can also be explained by the chemical composition of the methanolic extract where the major identified compounds such as linolenic acid and linoleic acid, vitexin, orientin and hydroxycinnamic acid have been reported to possess potent antioxidant properties [46, 47, 49, 52].

The antimicrobial activity of leaves extract of *H. muticus* L. subsp. *falezlez* revealed that the extract had good antibacterial activity against all tested strains. We also observed that the extract did not exert any antifungal effect against the tested strains. We compare our results with those obtained by other authors who have worked on species of the genus *Hyoscyamus*. In Algeria, Kebaili et al. [23] showed that the hydroalcoholic extract of *H. muticus* was effective on *E. coli* (12 ± 0.4 mm) and *S. aureus* (9 ± 1.12 mm) and was not effective on *P. aeruginosa*. Other studies that were conducted on *H. muticus* showed contrasting results. According to the study conducted by Elsharkawy et al. [15], the methanolic extract of the aerial parts of *H. muticus* showed significant inhibitory effects towards *S. aureus* and *Bacillus cereus*. They also noted a moderate inhibitory effect against *P. aeruginosa*, *E. coli* and *Klebsiella pneumonia*. The study by Al-Tohamy et al. [14] showed that *H. muticus* methanolic extract was ineffective on *S. aureus*, *K. pneumoniae* and *Aspergillus niger* and an inhibitory effect was observed on the yeast *C. albicans*. The ethanolic, chloroformic and hexane extracts of *H. muticus* leaves were effective on the following microbial strains: *Enterococcus faecalis*, *C. albicans*, *S. aureus*, *E. coli*, *P. aeruginosa* and *Salmonella Typhi* [37]. The extract of seeds of *H. muticus* from the study of Almalki [12] exhibited a moderate level of activity towards *B. subtilis* (MTCC 441), *E. faecalis* (ATCC 29212), *S. aureus* (ATCC 25923), *S. epidermidis* (MTCC 3615), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 15380) and *P. aeroginosa* (ATCC 27853). Furthermore, the same author reported a negative activity towards *A. niger* while the methanolic extract was active against several other fungi [12].

Our results of the antimicrobial activity can be justified by the chemical composition of the methanolic extract where we revealed the presence of molecules that were proved to have antimicrobial activity as palmitic, stearic and hydroxycinnamic acids and orientin [9, 44, 48]. Even though we reported the presence of linolenic acid and linoleic acid known for their antifungal activities in our extract, we did not find any antifungal activity.

5. CONCLUSIONS

The methanolic extract of *H. muticus* L. subsp. *falezlez* was investigated for its composition, antioxidant and antimicrobial properties. The studied extract was rich in phenolics and flavonoids. The HPLC and GC-MS analyses revealed the presence of various phytoconstituents, which are known for their diverse biological activities. The results also demonstrated that the methanolic extract exhibited potent antioxidant activity and can be used as a potential therapeutic agent in damage triggered by oxidative stress. The study indicated that the methanolic extract had antibacterial activity against the tested bacteria. Further investigations are needed for a better understanding of biomolecules contained in the methanolic extract and the mechanisms of action as antioxidant and antimicrobial agents.

Authors' Contributions: SA-G: methodology, data curation, formal analysis and writing original draft. ND, SS and SK: Data curation, formal analysis, review and editing. RG-T and FR: conceptualization, supervision, writing, review and editing. All authors read and approved the final manuscript.

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